Troubleshooting guide

Problem 1: Low sensor expression and/or poor transduction efficiency

Possible cause	***Solution***
Suboptimal sensor BacMam volume is being used.	Perform titration of the sensor BacMam stock, testing a large range (2.5-40 µl in 96-well plate format) to identify optimal volume. Too little can result in low expression, too much can cause cells to become sick.
Suboptimal cell density; too few or too many cells added.	Transduce cells so that the cells will be around 75-80% confluent at the time of transduction. Also, when transducing cells in suspension, make sure that cells in the source flask are < 100% confluent (approximately 80% confluent is ideal).
HDAC inhibitor was not added to the trans- duction mix, or the concentration was wrong.	Add HDAC inhibitor at the proper concentration: Sodium butyrate - 2mM Valproic acid - 5mM Trichostatin A - 0.25µM Perform a titration to determine optimal concentration for the cell type.
Cell type being used transduces poorly.	● After adding transduction mix to cells, let cells sit at room temperature for 30-40 min. before placing back in incubator (longer incubation times at room temperature may further improve transduction). ● Perform media exchange after various incubation times with the transduc- tion mix, in addition to leaving the virus on overnight. ● Try high-titer, purified BacMam stock. ● Validate assay in a different cell type (e.g. HEK 293T cells) ● Transduce cells multiple times (e.g. on Day 1, & again on Day 2). ● Incubate cells for 48 hours post transduction, before performing assay. ● Consider using a different viral vector, such as lentivirus or AAV.
Cell culture media is inhibiting transduction.	Remove media during transduction, preparing transduction mix in DPBS. Replace transduction mix with media after 2-4 hours.
BacMam stock was not stored properly (i.e. not stored at 4°C, exposed to light for long periods, subjected to multiple freeze-thaw cycles), or the shelf life has been exceeded.	Follow guidelines for product storage. BacMam stocks are stable for at least 12 months when stored properly. After this time period, the stock should be re-evaluated and compared to previous experiments. Purified BacMam stocks should be used within 30 days for best results.
BacMam stock was not mixed adequately before transducing cells.	Mix BacMam stock thoroughly before transduction, especially after being stored for long periods.
Promoter is not optimal for cell type being used.	Identify promoters that work best in the cell type being used.

Problem 2: Low fluorescence signal on microscope/plate reader.

Possible cause	***Solution***
Low sensor expression, low transduction efficiency.	See solutions for Problem 1 above.
Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the fluorescence spectra of the sensor. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelengths of the sensor.
Media exchange was not performed before running the assay; cells are in media rather than DPBS. Cell culture media being used has high autofluorescence.	Exchange media so that cells are in DPBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media.
Wrong microplate type is being used.	Use black, clear-bottom microplates with low autofluorescence.
Exposure time or gain setting on instrument is suboptimal.	Test different exposure and gain settings, monitoring how the signal-to-background and noise in the measurement changes. Too high can result in saturation and/or photobleaching; too low can result in noisy data.
Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. Confirm with visual inspection on a microscope.

Problem 3: Signal-to-background is low (i.e. cells/wells with sensor are not much brighter than control cells/wells without sensor).

Possible cause	***Solution***
Low sensor expression, low transduction efficiency.	See solutions for Problem 1 above.
Excitation/emission settings are not optimal for the sensor being used.	Refer to protocol for the fluorescence spectra of the sensor. Make sure that filter sets or monochromators are aligned with the peak excitation and emission wavelengths of the sensor.
Exposure time or gain setting on instrument is suboptimal.	Test different exposure and gain settings, monitoring how the signal-to-background and noise in the mea- surement changes. Too high can result in saturation and/or photobleaching; too low can result in noisy data.
Media exchange was not performed before running the assay; cells are in media rather than DPBS. Cell culture media being used has high autofluorescence.	Perform media exchange so that cells are in DPBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media.
Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. Confirm with visual inspection on a microscope.

Problem 4: Signal is noisy.

Possible cause	***Solution***
Low sensor expression, low transduction efficiency.	See solutions for Problem 1 above.
Gain setting or exposure time on instrument is too low.	Increase gain setting or exposure time.
Media exchange was not performed, or plate washing was inadequate causing high well-to-well variability. Cells are not in DPBS at the time of experiment.	Exchange media so that cells are in DPBS at the time of experiment. If cell culture media must be used, try using FluoroBrite media. Make sure that plate washing is highly consistent from well to well.
Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. Confirm with visual inspection on a microscope.

Problem 5: Good fluorescence signal, but sensor is not responding to drug as expected. No change in fluorescence observed, or signal is in the wrong direction.

Possible cause	***Solution***
Photobleaching	Reduce exposure time, sampling rate, and/or light intensity.
Drug is at the wrong concentration	Confirm drug concentration and solubility.
Drug was not stored properly.	Confirm drug storage conditions.
Drug was added to the cells in a volume that was too low relative to the volume of DPBS/media in the well, resulting in improper mixing.	Add drug in a volume that will allow for sufficient diffusion (i.e. 1:3 or 1:4 drug to total volume)
Drug was not added in the same solution as the solution in the well/culture dish.	Make sure drug preparation and cells are in the same solution.
Drug addition is producing an artifact.	Make sure to add a vehicle-only control. Make sure drug is added in a solution that is the same as the solution in the well. Do not exceed 1% DMSO final in the well (≤0.5% is ideal).
Compounds being tested are fluorescent.	Scan compounds for fluorescence to confirm. If possible, dilute compounds in order to reduce the fluorescence artifact of the compound.
Drug addition was too forceful and dislodged cells.	Add drugs manually or with an on-board dispense function, but do so gently, so as not to dislodge cells.
Baseline reads were not acquired before adding drug.	Acquire 5-10 baseline fluorescence reads before adding drug. Monitor for change in fluorescence intensity on adding drug.
Gain setting on instrument is too high, and signal is saturating. Gain setting is too low, and signal cannot be detected.	Adjust gain setting.
Too much sensor has been added to cells and the signal is saturated (i.e. not enough analyte for the amount of sensor in the cell).	Titrate the amount of sensor to determine maximum signal for your cell type.
Target receptor was not added, or expres- sion levels are suboptimal (too little or too much, or receptor has high level of constitutive activity).	Titrate the amount of receptor to optimize the signal for your cell type and receptor.
Sampling rate is not consistent with sensor kinetics.	Acquire 5-10 baseline measurements before adding drug. Resume measurement quickly after adding drug (within 5-10 sec for DAG/PIP2, 60 sec for cADDis, and 1-2 sec for GECO Ca2+). Measure long enough to capture max response.

Problem 6: Poor cell health, cells detaching from plate.

Possible cause	***Solution***
Too much BacMam stock was added to cells (e.g. sensor, receptor).	Titrate lower amounts of BacMam stock to identify the optimal volume for your cells.
Concentration of HDAC inhibitor is too high, or cells are sensitive to the HDAC inhibitor being used.	Confirm concentration of HDAC inhibitor being used. Make new stock solution. Try a different HDAC inhibitor. Confirm that they are being used at the proper concentration: Sodium butyrate - 2mM Valproic acid - 5mM Trichostatin A - 0.25µM Perform a titration to determine optimal concentra- tion for the cell type being used.
Plate surface is not coated with a cell attachment factor.	Coat plates with a cell attachment factor (e.g. PDL, laminin, collagen, fibronectin etc.) to enhance attachment.
Edge wells are being used, and cells in the edge wells may be subject to conditions that are not conducive to growth.	Do not use edge wells.
Cells were dislodged during media exchange/plate washing.	Make sure that media exchange or plate washing is done gently and does not dislodge cells. Confirm with visual inspection on a microscope.
DPBS being used does not contain calcium and magnesium.	Use DPBS containing calcium and magnesium.
Cells are contaminated.	Monitor cells for bacteria, fungi, mycoplasma.
Cells were not grown under proper growth conditions (i.e. 5% CO2, 37°C).	Incubate transduced cells at 37°C, in 5% CO2.